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14. ABSTRACT:
Cdk4 is an important regulator of G1/S cell cycle progression in mammalian cells. Loss of cdk4 in the mammary glands of mice results in abnormal development as evidenced by small fat pads and poor ductal branching. Deregulation of Ras signaling is seen in many cancers. The aim of this study is to determine the role of Cdk4 in Ras-induced breast tumorigenesis. Results presented in this study indicate that approximately 90% of cdk4 -/-: MMTV-v-Ha-ras mice remain resistant to v-Ha-ras-induced breast tumorigenesis while only 40% of their wildtype counterparts were found to be tumor-free. These studies also indicate that both cdk4 +/+: MMTV-v-Ha-ras mice and cdk4 (R24C/R24C): MMTV-v-Ha-ras mice induce Ras-driven breast tumors with the same frequency. Many of these tumors showed elevated levels of cell cycle proteins as well as increased levels and activity of Ras, Raf, MEK and ERK proteins. These results indicate that Cdk4 is important for v-Ha-ras-induced mammary tumorigenesis and that activating mutations in Cdk4 do not accelerate this process.

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Table of Contents

Introduction:

Breast cancer has second highest incidence of all types of cancer among women. Many proteins are deregulated in breast cancer, including cell cycle regulatory proteins (1). The gene encoding one of these proteins, *Cdk4*, is amplified in 16% of sporadic breast tumors and this amplification correlates with high Cdk4 protein levels (2). The Cdk4 (R24C) mutation is found in familial melanoma and results in the activation of Cdk4 kinase as it prevents the association of Cdk4 with its negative regulator, p16 (3) Mice that are homozygous for this mutation were shown to be susceptible to tumors of varying etiology, including those of the mammary gland (4).

Recently, it has been shown that the loss of *cdk4* in mammary tissues results in poor lobuloalveolar development and ductal branching (5). We and others have recently shown that Cdk4 is important for Neu (ErbB2/HER2)-induced breast tumorigenesis but not those induced by Wnt-1 (5, 6, 7). One of the downstream effectors of ErbB/HER signaling is Ras. ErbB/HER signaling often stimulates the Ras-Raf-MEK-ERK pathway (8). It has been shown that Ras requires cyclin D1 for breast tumor induction in mice (9). Since CyclinD1 complexes with Cdk4 and facilitates the progression of the G1 phase of the cell cycle, it was of interest to examine whether Cdk4 is required for v-Ha-ras-induced mammary tumors in mice and if the *cdk4* (R24C) mutation aggravates v-Ha-ras-induced breast tumorigenesis. The results of this study illustrate the importance of Cdk4 in Ras-mediated signaling in breast tumors and also expand our knowledge of different Ras signaling pathways in breast cancer.

Body:

Importance of Cdk4 in V-Ha-ras-induced mammary tumorigenesis: To gain an understanding of the role of Cdk4 in ras-induced breast tumorigenesis, *Cdk4(+/-)* and *Cdk4 (+/R24C)* mice were bred with MMTV-v-Ha-ras transgenic mice to generate *Cdk4(-/-)*:MMTV-v-Ha-ras, and *Cdk4(R24C/R24C)*:MMTV-v-Ha-ras mice, respectively. Since *Cdk4(-/-)* mice are infertile, we used *Cdk4(+/-)* mice for all matings. Whole mount and histopathological sections of the mammary glands derived from virgin adult mice (approximately 14 weeks) from these different crosses showed *Cdk4(+/+):*MMTV-v-Ha-ras mice exhibit proliferative disturbances in the mammary epithelium as evidenced by the appearance of multiple hyperplastic and dysplastic nodules that infiltrate the mammary fat pad (data not shown). Similar examination of whole mount and histopathological sections of mammary tissue derived from *Cdk4(-/-):*MMTV-v-Ha-ras mice showed very little or complete absence of any hyperplastic nodules. These results suggest that Cdk4 expression is essential for the appearance of MMTV-v-Ha-ras-induced proliferative disturbances. This is very similar to what was observed in *Cdk4(+/+):*MMTV-neu mice (5). Surprisingly, very little difference in the pathology of mammary tissue was seen between *Cdk4(+/+):*MMTV-v-Ha-ras mice and *Cdk4(R24C/R24C)*:MMTV-v-Ha-ras mice suggesting that onogenic mutations in the *Cdk4* gene contribute very little to the progression of breast cancer in the later mice.

Loss of expression of Cdk4 influences the incidence of mammary carcinomas in MMTV-v-Ha-ras transgenic mice. It has been previously reported that MMTV-v-Ha-

ras-induced breast carcinomas require the expression of cyclin D1 (9). To determine whether Cdk4 plays a similar role in the development of breast carcinomas induced by v-Ha-ras, we observed the four groups of transgenic mice for the appearance of breast tumors.

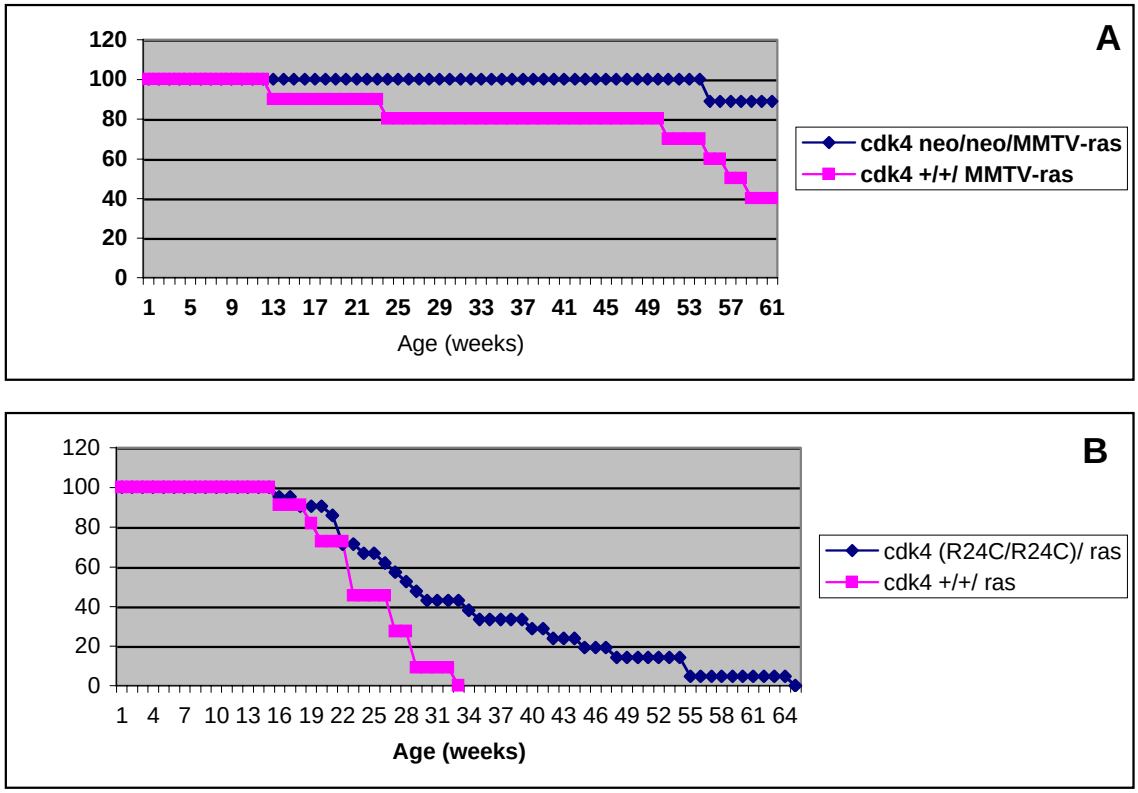

Figure 1. (A) Incidence of tumor formation in the mammary glands of *Cdk4-/-/*:MMTV-v-Ha-*ras* mice and their wildtype counterparts; (1B) A comparison of the incidence of breast tumor formation in Cdk4 (+/+):MMTV-v-Ha-ras mice and the *Cdk4(R24C/R24C)*:MMTV-ras counterparts.

The results of this study presented in **Fig. 1** show that approximately 60% of the *Cdk4(+/+):*MMTV-ras mice develop breast cancer between 12 to 60 weeks of age. In contrast, only about 11% of the *Cdk4(-/-):*MMTV-ras mice develop signs of breast cancer and this incidence was found to occur only after about 53 weeks of age. These observations suggest that development of breast tumors in MMTV-v-Ha-ras transgenic mice requires normal expression of Cdk4. As could be predicted from the histological examination of mammary tissues, very little difference in the development of mammary tumors was seen between *Cdk4(+/+):*MMTV-v-Ha-ras mice and *Cdk4(R24C/R24C)*:MMTV-v-Ha-ras mice. All of the *Cdk4(+/+):*MMTV-v-Ha-ras mice (100%) developed tumors in about 33 weeks. Similarly, 100% of the *Cdk4(R24C/R24C)*:MMTV-v-Ha-ras mice developed breast tumors in about 65 weeks, suggesting a lag in the tumor incidence in these mice. This is again surprising since the R24C mutation appears to influence tumor formation in a negative manner. Molecular mechanisms for this unexpected observation are currently being investigate

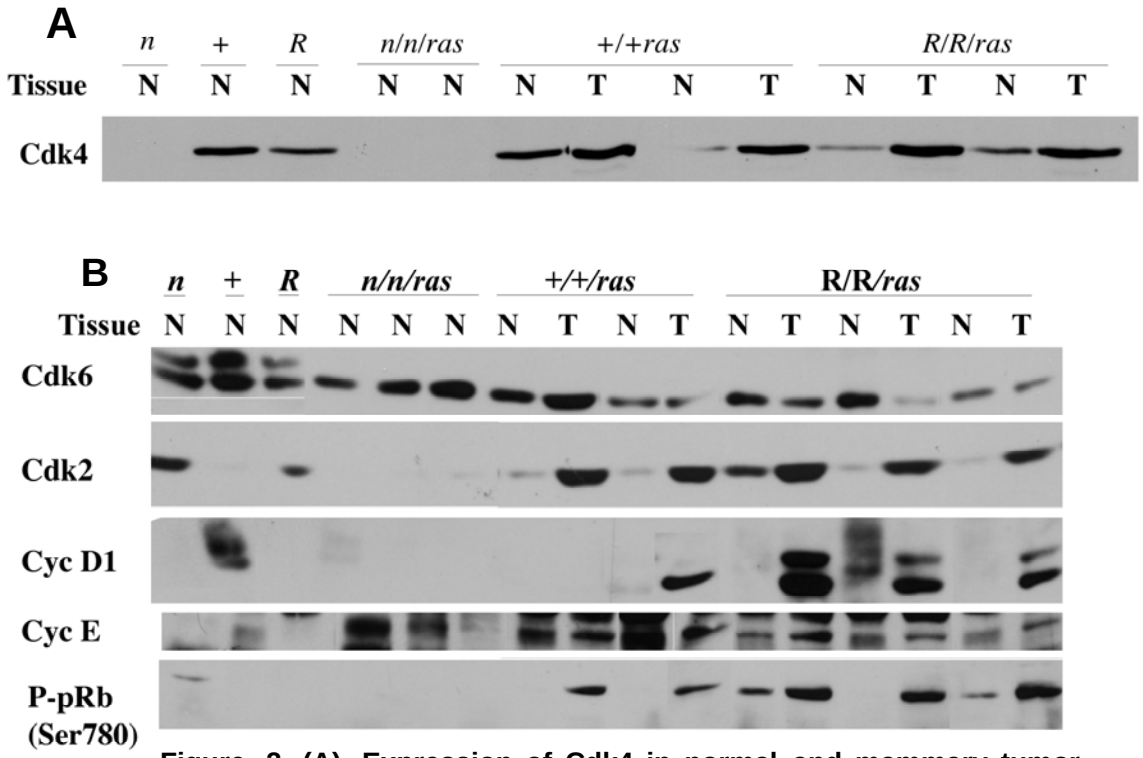

Figure. 2. (A). Expression of Cdk4 in normal and mammary tumor tissues of Cdk4-/- (n), Cdk4+/+ (+) and Cdk4R24C/R24C (R), Cdk4-/-: MMTV-v-Ha-ras (n/n/ras), Cdk4+/+: MMTV-v-Ha-ras (+/+/ras) and Cdk4R24C/R24C: MMTV-v-Ha-ras (R/R/ras). (B). Expression of Cdk6, Cdk2, Cyclin D1, Cyclin E and P-pRb (Ser780) in normal and mammary tumor tissues of Cdk4-/- (n), Cdk4+/+ (+) and Cdk4R24C/R24C (R), Cdk4-/-: MMTV-v-Ha-ras (n/n/ras), Cdk4+/+: MMTV-v-Ha-ras (+/+/ras) and Cdk4R24C/R24C: MMTV-v-Ha-ras (R/R/ras).

Analysis of cell cycle proteins: To determine the mechanisms associated with v-Ha-ras induced tumor development, we analyzed the expression of Cdk4, Cdk6, Cdk2, Cyclin D1, Cyclin E and phospho-Rb in the tumors and normal tissues derived from the *Cdk4* -/-:MMTV-*v-Ha-ras, Cdk4* (R24C/R24C):MMTV-*v-Ha-ras* as well as *Cdk4* +/+/MMTV-*v-Ha-ras* mice (**Fig. 2A**). As can be expected, results from these studies showed the absence of Cdk4 in the mammary tissues of Cdk4-/- mice. Abundant levels of this protein was seen in both normal and tumor tissues of Cdk4+/+ and Cdk4 (R24C/R24C) mice. Elevated expression of Cdk6 or Cdk2 was not seen in Cdk4-/- mice suggesting that loss of Cdk4 was not compensated through expression of Cdk6 or Cdk2. The results of these studies showed that Cdk6 levels were higher in the tumors of wild-type mice but were relatively lower in those of *cdk4* (R24C/R24C)/ MMTV-v-Ha-*v-Ha-ras* mice when compared to the normal mammary tissue of corresponding animals (**Fig. 2B**). There was an increased Cdk4-specific phosphorylation of pRb at Ser780 residue in the tumors of wild-type and *Cdk4* (R24C/R24C)/ MMTV-v-Ha-*ras* mice, which is in accordance with the expression of Cdk4 (**Fig. 2B**).

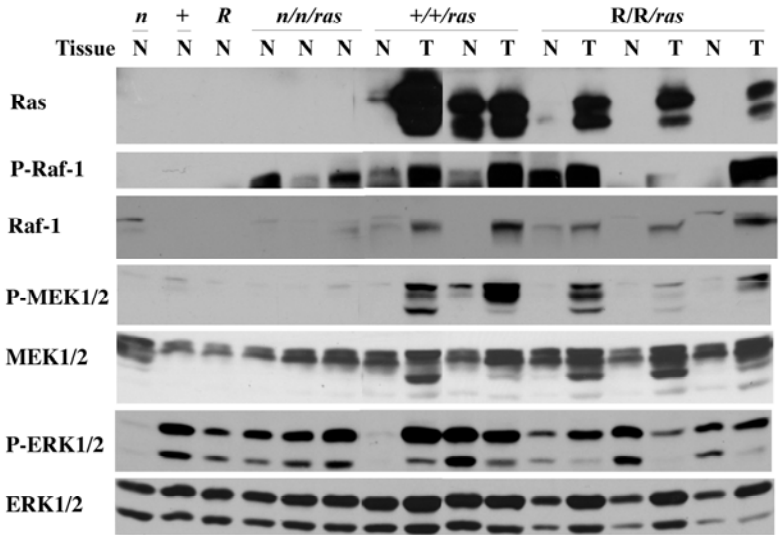

Figure. 3. Expression of Ras, Phospho-Raf1 (P-Raf1), Raf-1, Phospho-MEK1/2 (P-MEK1/2), MEK1/2, Phospho ERK1/2 (P-ERK1/2) and ERK1/2 in normal and mammary tumor tissues of Cdk4-/- (n), Cdk4+/+ (+) and Cdk4R24C/R24C (R), Cdk4-/-: MMTV-v-Ha-ras (n/n/ras), Cdk4+/+: MMTV-v-Ha-ras (+/+/ras) and Cdk4R24C/R24C: MMTV-v-Ha-ras (R/R/ras).

Analysis of the Ras-Raf-MEK-ERK pathway: Previous studies have shown that Ras requires cyclin D1 for breast tumor induction in mice (9). As cyclin D1 complexes with Cdk4 and promotes cell cycle progression through the G1 phase, it was of interest to determine the level of Ras expression in the mammary glands and tumors of our Ras/Cdk4 animal models. As expected, upregulation of Ras expression was observed in the tumors of *cdk4* (R24C/R24C): MMTV-*v-Ha-ras* and *cdk4 +/+/*MMTV-*v-Ha-ras* mice. To further dissect this pathway, we analyzed the expression of downstream signaling proteins of the Ras pathway such as Raf-1, MEK1/2 and ERK1/2. Study of Raf-1, MEK1/2, ERK1/2 proteins indicated increased activity as well as increased expression in many of the tumors (Fig. 3).

Relationship to previous findings. In the previous report, I presented data which showed that Cdk4 expression is essential for ErbB2/Neu induced breast tumorigenesis. but not for Wnt-1- induced breast tumorigenesis. Results presented in this report further suggest that downstream effctors of ErbB2/Neu such as ras also require the expression of Cdk4 for the manifestation of their tumorigenic activity.

Key research accomplishments:

1. Our results presented in this report show that expression of Cdk4 is important for v-Ha-ras-induced mammary tumorigenesis.

2. Activating mutations in the Cdk4 gene such as the R24C do not accelerate v-Ha-ras-induced tumorigenesis.

3. One of the pathways v-Ha-ras signals through in Ras-induced tumorigenesis is the Raf-MEK-ERK pathway.

Reportable outcomes:

None

Conclusions:

1. Results presented in this study indicate that approximately 90% of *cdk4 -/-*: MMTV-v-Ha-*ras* mice, remain resistant to Ras-induced breast tumorigenesis while only 40% of their wildtype counterparts were found to be tumor-free.

2. Our studies indicate that both *cdk4 +/+*: MMTV-v-Ha-*ras* mice and *cdk4* (R24C/R24C): MMTV-v-Ha-*ras* mice induce Ras-driven breast tumors with the same frequency.

3. Increased activity as well as elevated levels of Ras, Raf, MEK and ERK proteins in many of these tumors indicate that one of the pathways Ras signals through in these tumors is the Ras-Raf-MEK-ERK pathway.

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